SUPPLEMENTAL MATERIAL

1. **Codex Microbiological Criteria for *Listeria monocytogenes* in Ready-To-Eat Foods**
2. **European Commission Regulations Dedicated for Food Business Operators**
3. **Establishing Shelf-life with Respect to *L. monocytogenes***
4. **European Union Definitions**
5. **Performance of Sampling Plans**
6. **Frequency of Sampling Plans**
7. **Listeria Sampling and Monitoring**
8. **Estimation of Listeriosis Cases Resulting from the Consumption of Uncooked Frozen Vegetables in Elderly Men (U.S.)**
9. **References**

1. Codex and European Union (EU) microbiological criteria for *Listeria monocytogenes* in ready-to-eat (RTE) foods:

a) Codex microbiological criteria for RTE foods in which growth of L. monocytogenes will not occur:

For RTE foods in which the growth of *L. monocytogenes* will not occur, various factors such as the pH and a_w, were considered in developing the rationale for the policy. Growth limits for *L. monocytogenes* were stated as being a pH value < 4.4, an a_w value of < 0.92, or a combination of factors (pH, a_w), e.g., the combination of a pH < 5.0 and an a_w < 0.94. In addition, frozen products fall into the category of foods that do not support growth of the organism. (Codex, 2007)

Table 1a.

Point of Application	Microorganism	n	c	m	Class Plan
Ready-to-eat foods from the end of manufacture or port of entry (for imported products, to the point of sale	*Listeria monocytogenes*	5[a]	0	100 cfu/g[b]	2[c]

Where n=number of samples that must conform to the criterion; c=the maximum allowable number of defective sample units in a 2-class plan; m=a microbiological limit which, in a 2-class plan separates acceptable lots from unacceptable lots.

[a] National government(s) should provide or support the provision of guidance on how samples should be collected and handled, and the degree to which compositing of samples can be employed.

[b] This criterion is based on the use of the ISO 11290-2 method.

Other methods that provide equivalent sensitivity, reproducibility, and reliability can be employed if they have been appropriately validated, e.g., based on ISO 16140 (ISO, 2017)

[c] Assuming a log- normal distribution, this sampling plan would provide 95% confidence that a lot of food containing a geometric mean concentration of 93.3 cfu/g and an analytical standard deviation of 0.25 log cfu/g would be detected and rejected if any of the five samples exceeding 100 cfu/g *L. monocytogenes*. Such a lot may consist of 55% of the samples being below 100 cfu/g and up to 45% of the samples being above 100 cfu/g, whereas 0.002 % of the samples from this lot could be above 1000 cfu/g.

The typical actions to be taken where there is a failure to meet the above criterion would be to (1) prevent the affected lot from being released for human consumption, (2) recall the product if it has been released for human consumption, or (3) determine and correct the root cause of the failure.

b) Codex microbiological criteria for RTE foods in which growth of L. monocytogenes can occur:

Codex has defined a food supporting the growth of *L. monocytogenes* as a RTE food in which there is greater than an average of 0.5 log cfu/g increase in the levels of *L. monocytogenes* for at least the expected shelf life of the food, under reasonably foreseeable conditions of distribution, storage and use (Codex, 2007).

The criterion seen in Table 2 below is intended for foods in which the growth of *L. monocytogenes* can occur under the conditions of storage and use that have been established for the product. This Codex criterion is based on the product being produced under GMPs and HACCP, with appropriate evaluation of the production environment and process control. The whole purpose of this Codex criterion was to provide companies operating in Codex member countries, a specified degree of confidence that *L. monocytogenes* will not be present in foods at levels that represent a risk to consumers.

Table 1b.

Point of Application	Microorganism	n	c	m	Class Plan
Ready-to-eat foods from the end of manufacture or port of entry (for imported products), to the point of sale	*Listeria monocytogenes*	5[a]	0	Absence in 25g (<0.04 cfu/g)[b]	2[c]

[a] National government(s) should provide or support the provision of guidance on how samples should be collected and handled, and the degree to which compositing of samples can be employed.

[b] Absence in a 25-g analytical unit. This criterion is based on the use of the ISO 11290-1 method. Other methods that provide equivalent sensitivity, reproducibility, and reliability can be employed if they have been appropriately validated, e.g., based on ISO 16140 (ISO, 2017).

[c] Assuming a log- normal distribution, this sampling plan would provide 95% confidence that a lot of food containing a geometric mean concentration of 0.023 cfu/g and an analytical standard

deviation of 0.25 log cfu/g would be detected and rejected if any of the five samples exceeding 100 cfu/g *L. monocytogenes*. Such a lot may consist of 55% of the 25g samples being negative and up to 45% of the 25 g samples being positive. 0.5 % of this lot could harbor concentrations above 0.1 cfu/g.

The typical actions to be taken where there is a failure to meet the above criterion would be to (1) prevent the affected lot from being released for human consumption, (2) recall the product if it has been released for human consumption, or (3) determine and correct the root cause of the failure.

2. European Commission regulations dedicated for food business operators

This legislation requires food business operators (FBOs) to:

- Decide whether the product is ready to be consumed as such, without the need to cook or otherwise process it to ensure its safety and compliance with the microbiological criteria. (RTE definition), and
- Conduct studies in accordance with Annex II of the Regulation to investigate compliance with the criteria throughout shelf life. This particularly applies to RTE ready-to-eat foods that are able to support the growth of *L. monocytogenes* and that may pose a *L. monocytogenes* risk for public health. Food businesses may collaborate in conducting those studies.
- Use validated analytical methods. If using other than the stipulated reference methods (detection EN/ISO 11290-1, enumeration EN/ISO 11290-2), alternative methods must be shown to provide equivalent results, i.e., are validated according to EN/ISO standard 16140 or other internationally accepted similar protocols (ISO, 2017).

The legislation also requires food business operators (FBOs) to carry out sampling and testing of:

- RTE foods where *L. monocytogenes* is a hazard organism when validating or verifying the correct functioning of HACCP-based procedures and Good Hygiene Practice, and ii)
- Processing areas and equipment where *L. monocytogenes* is a hazardous organism, sampling in accordance with ISO 18593 (ISO, 2018)

The Regulations do not stipulate the frequency of sampling for *L. monocytogenes* but require FBOs to:

- Sample foods as part of their procedures based on HACCP principles of Good Hygiene Practice, taking into account the instructions for use of the foodstuff, and the nature and size of the food businesses, provided that the safety of foodstuffs will not be endangered, and
- Perform trend analysis results and, on finding of exceedances of criteria set for foods, and adverse environmental data trends, pursue action without undue delay to find the cause of unsatisfactory results and implement corrective action to prevent recurrence.

Samples taken from a given process under practically identical circumstances and produced in a specific place within one defined production period can be composited together. Other sampling

and testing procedures (e.g., alternative sampling sites and use of trend analyses) can be used by FBOs if they can demonstrate to the satisfaction of the competent authority, that these procedures provide at least equivalent guarantees.

The European Union Reference Laboratory (EURL) for *L. monocytogenes* in 2012 issued updated Guidelines on sampling the food processing area and equipment for the detection of *Listeria monocytogenes* (Version 3 – 20/08/2012) (ANSES, 2017). This Guidance covers:

- Choice of sampling locations
- Time at which sampling should be performed
- Diluents to moisten the wipe sampling devices
- Area to be sampled
- Preparation of sampling devices
- Sampling methods
- Transport and storage of samples
- Sample analysis
- Expression of results

Food Usage Labelling

European Council Directive (79/112/EEC) (EU Regulations, 1978) on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs for sale to the ultimate consumer (79/112/EEC), made mandatory provision of instructions for use of a foodstuff on the labelling when it would be impossible to make appropriate use of the foodstuff in the absence of such instructions.

This requirement is retained in the most recent iteration of EU food labelling legislation, Regulation 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers (EU Regulations, 2011) . EU Regulation 2073/2005 states that such instructions should be taken into account by FBOs when deciding appropriate sampling frequencies for the testing against microbiological criteria (EU Regulations, 2005).

Table 2. *Listeria monocytogenes* Food Safety Criteria from EC Regulation 2073/2005

a) Ready-to-eat Foods Intended for Infants and Ready-to-eat Foods for Special Medical Purposes		
Listeria monocytogenes	n=10 c=0 m= not detected/absence in 25g (EN/ISO 11290-1)	Products placed on the market during their shelf-life.
b) Ready-to-eat Foods Able to Support the Growth of *L. monocytogenes*, Other Than Those Intended for Infants and for Special Medical Purposes		
Listeria monocytogenes	n=5 c=0 m= 100 cfu/g (EN/ISO 1120-21)	Products placed on the market during their shelf-life.
	n=5 c=0 m= not detected/absence in 25 g (EN/ISO 1120-1)	Before the food has left the immediate control of the food business operator, who produced it.
	c=0 m= 100 cfu/g (EN/ISO 11290-2)	
c) Ready-to-eat Foods Unable to Support the Growth of *L. monocytogenes*, Other Than Those Intended for Infant and for Special Medical Purposes		
Listeria monocytogenes	n=5 c=0 m=100 cfu/g (EN/ISO 11290-2)	Products placed on the market during their shelf-life

A number of food type exclusions are listed in the regulation under the criterion:

- those which have received heat treatment or other processing effective to eliminate *L. monocytogenes*, when recontamination is not possible after this treatment (e.g., products heat-treated in their final package)
- fresh, uncut and unprocessed vegetables and fruits, excluding sprouted seeds
- bread, biscuits and similar products
- bottled or packed waters, soft drinks, beer, cider, wine, spirits and similar products
- sugar, honey and confectionery, including cocoa and chocolate products
- live bivalve molluscs
- food grade salt

In addition, the regulation lists products that will not support the growth of *L. monocytogenes* as including foods with:

- a pH $\leq$ 4.4 or
- an $a_w \leq 0.92$
- a shelf life of less than 5 days
- a pH $\leq$ 5.0 and an $a_w \leq 0.94$

Frozen foods at less than -12°C will not support the growth of *L. monocytogenes*.

3. Establishing shelf-life with respect to *L. monocytogenes*

EC No 2073/20052073/2005 requires that RTE foods must not exceed the limit of 100 CFU/g for *L. monocytogenes* at any point during their shelf life (except those intended for infants or particular medical purposes, which must not contain *L. monocytogenes* in 10 samples of 25g). Otherwise *L. monocytogenes* must be absent in 5 x 25g samples at the point of manufacture. For an FBO to apply the 100 CFU/g limit, they must have evidence for each product to show that *L. monocytogenes* does not exceed 100 CFU/g throughout the shelf life (EU Regulations, 2005).

Annex II of 2073/2005 gives a series of options available to manufacturers to determine how *L. monocytogenes* would behave in a product, and this should be used in 'studies' to establish shelf life:

- Specifications for physico-chemical characteristics of the product, such as pH, a_w, salt content, concentration of preservatives and the type of packaging system, taking into account the storage and processing conditions, the possibilities for contamination and the foreseen shelf-life, and
- Consultation of available scientific literature and research data regarding the growth and survival characteristics of the microorganisms of concern.
- When necessary on the basis of such studies, the FBO shall conduct additional studies, which may include:
    - Predictive mathematical modelling established for the food in question, using critical growth or survival factors for the microorganisms of concern in the product,
    - Tests to investigate the ability of the appropriately inoculated microorganisms of concern to grow or survive in the product under different reasonably foreseeable storage conditions,
    - Studies to evaluate the growth or survival of the microorganisms of concern that may be present in the product during the shelf-life under reasonably foreseeable conditions of distribution, storage and use.

These studies are required to take into account the inherent variability linked to the product, the microorganisms in question and the processing and storage conditions. FBOs can collaborate in

conducting these studies. FBOs must also keep documentation of shelf life studies and verification as part of GMP and HACCP procedures.

Protocols for shelf-life evaluation (e.g., Evaluation of Product Shelf life for Chilled Foods) are available which provide a basis for historical data sets. Using historical data can provide the best evidence to demonstrate consistent control of the level of *L. monocytogenes* in a particular food. Historical data on levels of *L. monocytogenes* in existing RTE foods at the start and/or end of shelf life can be used to assess its growth potential and confirm that the assigned shelf life is appropriate. It can also be applied to similar RTE foods with comparable intrinsic characteristics (pH, a_w, microflora, etc.) produced under practically identical conditions. These should be specific to the FBO's premises and foods; however, collaboration between FBOs is acceptable either between different sites within the same company or different companies, e.g., through a trade association. The FBO should be able to demonstrate to an enforcement officer that the products and the processing of the products for which the data are being shared are similar.

For example:

- For these studies to be valid the products being compared should have the same characteristics (pH, a_w, salt content, concentration of preservatives, type of packaging, associated microflora or any other characteristic important for the survival and growth of *L. monocytogenes*), and;
- The production process and storage conditions of the products should be similar.

It must be noted that different production areas will have different potential for contamination. However, products may have the same potential for growth of *L. monocytogenes* if contaminated. If the products are not similar, the FBO should be able to show how they are different and what effect those differences have on the survival and growth of *L. monocytogenes*.

Data to be used to verify shelf life should include information from HACCP and monitoring checks, including:

- Process validation, verification and monitoring (e.g., temperature, time, pH and a_w)
- Ingredients traceability and microbiological quality testing including for hygiene indicator organisms and/or *L. monocytogenes*
- Sampling for *Listeria* species and appropriate hygiene indicator organisms from processing areas and equipment (to demonstrate the efficacy of factory hygiene and cleaning regimes)

- Final product testing for *L. monocytogenes* for example on the day of production and/or at the end of shelf life to verify effective functioning of the HACCP system and durability verification

Manufacturers of RTE foods should understand how *L. monocytogenes* behaves in their product, as determined by the products formulation, storage conditions, packaging and shelf life. The factors deemed to be critical in controlling development of *L. monocytogenes* must be identified, controlled and monitored (e.g., in a hard cheese, salt, moisture, pH and acidity are likely to be the most significant factors restricting growth of *L. monocytogenes*). Tolerances and limits for these parameters should be specified and controlled.

4. European Union (EU) definitions

- Food safety criterion: a criterion defining the acceptability of a product or a batch of foodstuff applicable to products placed on the market
- Process hygiene criterion: a criterion indicating the acceptable functioning of the production process. Such a criterion is not applicable to products placed on the market. It sets an indicative contamination value above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law;
- Batch: a group or set of identifiable products obtained from a given process under practically identical circumstances and produced in a given place within one defined production period;
- Shelf-life: either the period corresponding to the period preceding the ‘use by’ or the minimum durability date (best before)
- Ready-to-eat food: food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level of microorganisms of concern
- Food intended for infants’ means food specifically intended for infants
- Food intended for special medical purposes: dietary food for as defined in Commission Directive 1999/21/EC, which has since been replaced by Regulation (EU) No 609/2013 (EU Regulations, 2013).

5. Performance of sampling plans

Table 6 in the main paper provides additional results to reflect on the use of alternate standard deviations (0.25, 0.4 and 1.2) and calculations of sampling performance, expressed as a function of the geometric mean. The general conclusions are similar, regardless of whether the geometric or arithmetic means are used.

Effect of high background levels of L. innocua on the recovery of L. monocytogenes (Table 1)*:* If *Listeria* spp. is detected and, for example, 5 colonies of a plate are identified and found to be *L. innocua*, it is very dangerous to conclude that no *L. monocytogenes* was present. For example, due to the low power of this test (only 5 colonies tested), if 10% of the colonies were *L. monocytogenes,* there is a 59% probability of having only *L. innocua* as the test result.

Table 3. The performance of identifying 5 colonies from a *Listeria* spp. plate depending on the proportion of *L. monocytogenes* and *L. innocua.*

% *L. monocytogenes* present	*P (L. innocua)*	*P* (5 times *L. innocua*)
50	0.5	0.03
20	0.8	0.33
10	0.9	0.59
5	0.95	0.77

Furthermore *L. innocua* can outgrow *L. monocytogenes* in Frasier Broth (Cornu et al., 2002); Gnanou-Besse et al., 2005; Zitz et al., 2011), so *L. monocytogenes* can be initially present at a high proportion but be overgrown by *L. innocua* in the enrichment. Therefore, it is better to test use a qPCR method specific for L. monocytogenes directly from the enrichment, since much lower levels of *L. monocytogenes* can be detected in a background of *L. innocua*, rather than with selective plating.

Adaptation of the ICMSF spreadsheet

To be able to calculate the performance of mixed qualitative/quantitative plans, the ICMSF spreadsheet was adapted.

Legan et al. (2001) described the procedure for quantitative 2- and 3-class sampling plans, using a lognormal distribution of the concentration in the food to describe the probability that a sample will have a logC below *m* (P_a) or between *m* and *M* (P_m). With these values the probability of accepting a sampling plan with a given *n* and *c* value is then calculated (using the binomial distribution). This was implemented in the first version of the ICMSF sampling tool. Van Schothorst et al. (2009) described the use of the Poisson-Lognormal distribution to describe the P_a for a presence/absence test, given a certain sample weight, which was then implemented additionally to the ICMSF sampling tool.

For this research the tool was further extended to include 3-class plans having a quantitative upper limit but with an *m* based on presence/absence.

For a quantitative 3-class plan P_a and P_m are calculated by

P_a= NORMSDIST((*m*-logC)/sigma)

P_m= NORMSDIST((*M*-logC)/sigma)-NORMSDIST((*m*-logC)/sigma) = NORMSDIST((*M*-logC)/sigma)-P_a

In the mixed 3-class plan the formulas are changed in the following manner:

P_a= 1-PoissonLogNormal(logC, sigma, sample weight)

P_m= NORMSDIST((*M*-logC)/sigma)-P_a

Where PoissonLogNormal is calculated by

$$Poissonlognormal(\mu_{logC}, \sigma, weight) = \int_{-\infty}^{\infty} Pnormal(logC, \mu_{logC}, \sigma)\left(1 - Poisson(0, 10^{logC} \cdot weigth)\right) dlogC$$

6. Frequency of sampling

If a microbiological criterion exists, one should follow the exact criterion (e.g., n= 5 samples of m= absence in 25g) when sampling. For certain criteria, the frequency is specifically indicated in legislation, and then this has to be followed. For other criteria, there is usually no specification for the frequency of testing. In principle, the producer should have the appropriate controls in place such that the criteria are in compliance. However, if the frequency of sampling is not indicated specifically by legislation, one could do sampling of every batch, but one could also select another frequency.

In addition, a government can perform surveillance testing or a baseline study, and then decide to test more batches or more products with n=1 instead of a higher n value (as set in the criteria) to have broader coverage.

In EU Directive 2073/2005 (EU Regulations, 2005), it is stated: "Food business operators shall decide the appropriate sampling frequencies, except where Annex I provides for specific sampling frequencies, in which case the sampling frequency shall be at least that provided for in Annex I. Food business operators shall make this decision in the context of their procedures based on HACCP principles and good hygiene practice, taking into account the instructions for use of the foodstuff.

The frequency of sampling may be adapted to the nature and size of the food businesses, provided that the safety of foodstuffs will not be endangered."

In the European Commission regulation EU 2073/2005, examples of sampling plans with indicated frequency are provided (EU Regulations, 2005).

The food business operators of slaughterhouses or establishments producing minced meat, meat preparations, mechanically separated meat or fresh poultry meat shall take samples for microbiological analysis at least once a week. The day of sampling shall be changed each week to ensure that each day of the week is covered.

- As regards the sampling of minced meat and meat preparations for *E. coli* and aerobic colony count analyses and the sampling of carcasses for Enterobacteriaceae and aerobic colony count analyses, the frequency may be reduced to fortnightly testing if satisfactory results are obtained for 6 consecutive weeks.
- In the case of sampling for *Salmonella* analyses of minced meat, meat preparations, carcasses and fresh poultry meat, the frequency may be reduced to fortnightly testing if

satisfactory results have been obtained for 30 consecutive weeks. The *Salmonella* sampling frequency may also be reduced if there is a national or regional *Salmonella* control program in place and if this program includes testing that replaces the sampling laid down in this paragraph. The sampling frequency may be further reduced if the national or regional *Salmonella* control program demonstrates that the *Salmonella* prevalence is low in animals purchased by the slaughterhouse.

- However, when justified on the basis of a risk analysis and consequently authorized by the competent authority, small slaughterhouses and establishments producing minced meat, meat preparations and fresh poultry meat in small quantities, may be exempted from these sampling frequencies.
- Food business operators producing sprouts should take samples for microbiological analysis at least once a month at the stage where the probability of finding Shiga-toxin producing *E. coli* (STEC) and *Salmonella* spp. is the highest, and in any case not before 48 h after the start of the sprouting process.

7. Listeria sampling and monitoring

The need for sampling

Analyzing *Listeria spp.* and *L. monocytogenes* data on food and production environment enables businesses to establish appropriate product shelf lives and control production hygiene.

European Commission legislation (2073/2005) requires food business operators to gather, trend and react to such data to ensure control is maintained where *L. monocytogenes* is a hazard (EU Regulations, 2005). A continuous data stream from food and production environment sampling is therefore required, being analyzed on an ongoing basis, with corrective action carried out and its efficacy verified.

Regular programmed food and environmental sampling must be carried out according to established protocols designed to determine the prevalence of *Listeria* spp. and *L. monocytogenes* in food and the production environment, and *L. monocytogenes* levels in food.

- Sampling must in both cases be designed to actively seek out *L. monocytogenes* primarily to enable its control to be assessed and further improved to protect food safety, and also to demonstrate regulatory compliance.
- The identification of a positive *Listeria spp.* from food or the production environment should always be followed up by confirmation of the presence or absence of *L. monocytogenes*.
- All positive findings must be investigated (i.e., root cause analysis); corrective actions should be applied; and efficacy be verified.
- Clear targets and standards must be implemented consistently and robustly for suppliers and internally for operational and hygiene teams. Targets must be specific about whether they apply to *Listeria species* or specifically *L. monocytogenes*.
- Sampling plans for environmental monitoring, raw materials, process control or finished product should be agreed upon, published and communicated to key establishment personnel prior to production.
- An adverse trend is when levels (of what?) are frequently at or near the Report level, or when a significant increase over the level normally observed is seen.
- Adverse trends must be identified, investigated and actioned. Documentation of the actions taken must be kept and corrective actions must be verified as being effective.

- Microbiological performance in relation to key indicator organisms including *Listeria spp.* and *L. monocytogenes* in ready-to-eat (RTE) foods should be a site key performance indicator (KPI) and be reported to and discussed with senior management level and addressed effectively.
- The escalation procedure where results are above target levels for *Listeria* spp. or *L. monocytogenes* should be agreed upon and communicated clearly to those responsible for responding to the data, both internally within the food business and, where appropriate, to the brand owner if not the manufacturer.
- Company action plans such as targeted and/or increased sampling should be agreed upon in advance where positive results are reported. Agreed procedures and responsibilities should be in place.
- Root cause analysis must be carried out if the cause of a positive result has not previously been identified. Corrective actions must be put in place as soon as practicable to minimize the potential for a recurrence of contamination.

Food Sampling

Food businesses should be able to demonstrate to the satisfaction of competent authorities that their products, when properly handled and stored during distribution, retail and by consumers, meet the food safety criteria throughout the shelf life. To this end, the food business may need to conduct studies to investigate compliance during shelf life.

The EU Microbiological Criteria for Foodstuffs Regulation EC 2073/2005 sets out in Article 3.2 and Annex II the recommended approach to shelf life assessment (EU Regulations, 2005). The emphasis is on standard shelf life assessment testing, taking into account the storage and processing conditions and available scientific literature and research data regarding the growth and survival characteristics of the microorganisms of concern. Historical data is therefore of particular value.

Secondary tools such as pathogen modelling and challenge testing may also be used, but there are practical restrictions as to their applicability. Since most prepared products are not homogeneous, it is important to understand the source data of the model being used, i.e., whether the organism was grown in broth or real food. In addition, processed foods often contain stressed cells, which need to be taken into account.

A food business operator having implemented GMP, HACCP and supporting systems and following the shelf life assessment approach set out in Annex II of 2073/2005 is not expected to have to carry out *L. monocytogenes* challenge testing.

The shelf life of foods is not only limited by microorganisms. Often, enzymatic, physical or chemical changes or a combination of these limit shelf life. Spoilage of chilled products can often be detected earlier by visual and other organoleptic means rather than by testing.

HACCP, with the assistance of microbiological risk assessment, is used to control safety during product development, at which point the whole of the supply chain is considered and pathogens which are not generally tested for, are controlled. Pathogen testing and challenge testing as part of shelf life is therefore of little relevance in a well-designed product made under controlled conditions. The safety/stability of a product should instead be addressed during new product development wherever possible.

Compositing of samples across comparable batches of products may be appropriate, e.g., for the detection of *L. monocytogenes* in identifiable products obtained from a given process under practically identical circumstances and produced in a given place with one defined production period, as set out in 2073/2005. (see also section on 'Alternative Approaches to Current Two-Class Sampling Plans' in the manuscript).

Historical data provides a very good indication of the behavior of an organism in a particular food. Data on the levels of *L. monocytogenes* present at the beginning and at the end of shelf life can be used to assess its potential for growth.

For example, if *L. monocytogenes* was detected in a RTE cooked meat product at the beginning of shelf life at a level of <20 CFU/g, and end of life data on a number of separate representative samples from the same batch showed levels of no more than 100 CFU/g, then the data helps demonstrate that from a *L. monocytogenes* perspective that the product remains within the food safety criteria set out in 2073/2005 over its shelf life. Under such circumstances, a low level (<20 CFU/g) detection during shelf life would not require the product to be withdrawn in most countries, unless the product has been associated with illness or was produced under unacceptable GMPs. This approach is the most valid providing that end of life samples have

either followed the normal route of distribution, storage and retail, (e.g., sampling from the shelf for retail products) or have been stored at temperatures closely simulating those conditions.

The limitation of this method is that for most of the time *L. monocytogenes* should be absent in the foodstuff; it can therefore be difficult or take time to acquire such data. It also provides no information on safe shelf life for new products, particularly if a new product is introduced that is significantly different from those usually produced at the manufacturing site. Manufacturers should therefore (under 2073/2005) construct a database for *L. monocytogenes* consisting of appropriate samples taken at the beginning and end of life for each ready-to-eat product. In the UK, the Chilled Food Association established such a database prior to implementation of 2073/2005. The dataset now comprises more than 2.5 million data points from its members' sampling of food and the production environment.

Under 2073/2005, if there is evidence that *L. monocytogenes* will not exceed 100 CFU/g during shelf life, finding *L. monocytogenes* at lower levels is not required to be notified to the Competent Authority. However, internal company action is required including when exceeding the Process Hygiene Criteria in Annex I, Chapter 2 of 2073/2005 and on discovery of adverse monitoring trends (EU Regulations, 2005).

Internal actions will include:

- Traceability of the sample
- Review of microbiological testing results
- Investigate common influences
- Monitoring at key points to establish the source of contamination or the breakdown of the process, e.g., equipment and external influences such as water quality issues
- Implement corrective action
- Verify that the corrective action has been successful; this may include increased sampling

In the case of a food safety issue arising, timely action must be taken to protect consumer safety. Actions required relate to Article 19 of EU General Food Law Regulation 178/2002/EC and include (EU Regulations, 2002):

- In the case of own label product manufacture, immediate liaison with the brand owner.

- Brand owner, i.e., food manufacturer or retailer in the case of a retailer's own label products to notify the Competent Authority and recall final product. The extent of the problem must be made clear. For example:
    - Restricted to a particular batch/size/distribution area
    - Reassure that all other batches/sizes/products are safe
    - Number of packages involved
    - Speed and efficiency of recall
    - Cause of fault being investigated
- Remedial actions in the supply chain should involve the consideration of:
    - What to do to re-establish control and prevent reoccurrence of the hazard?
    - What to do with product and raw material held in stock or in the supply chain that might be out of specification?
    - When the action taken should be completed? i.e. the timescale for the action
    - Who has responsibility for action?

Environmental Monitoring

It is important to note that finding *Listeria spp.* on a product contact surface does not automatically mean that product is contaminated. However, the nature of potentially exposed product must be determined considering the likelihood of transfer, the ability of the product to support growth and its intended use.

Environmental sampling is required by EU regulation 2073/2005 to be carried out on food contact and non-food contact surfaces and can be used for the following:

- Verifying hygienic status following commissioning or maintenance of plant.
- Verifying the efficacy of cleaning and disinfection immediately before start-up of production or following production cleaning.
- Monitoring the hygienic status of the environment during production.
- Investigating source/s and vector/s of contamination (these data should not be used in trend monitoring)
- Revalidation of cleaning and disinfection.

Article 5 (paragraph 2) of 2073/2005 requires that:

- Samples shall be taken from processing areas and equipment used in food production, when such sampling is necessary for ensuring that the criteria are met. In that sampling, the ISO standard 18593 shall be used as a reference method (ISO, 2018);
- Food business operators manufacturing ready-to-eat foods, which may pose a *L. monocytogenes* risk for public health, shall sample the processing areas and equipment for *L. monocytogenes* as part of their sampling scheme.

A comprehensive, robust and dynamic environmental monitoring program is essential where there is a risk of *L. monocytogenes* contamination of RTE foods being designed to detect and therefore facilitate eradication of the organisms. For any sample site, areas of obvious dirt, grease or condensate should be swabbed rather than obviously clean areas, otherwise results may give a false sense of security.

The environmental monitoring program should include:

- Facility-Specific Risk Assessment
- Developing a Sampling Plan
    - What microorganisms (*Listeria* spp. or other indicator microorganisms) to monitor(CFA and BRC, 2006)
    - Sampling frequency.
    - Number of samples to be collected.
    - Where and when samples will be collected.
    - Sampling and testing methods
- Results Tracking and Trending
- Response to Results and Corrective Actions
- Special Considerations
- Program Verification and Documentation
- What if *Listeria* spp. Is Never Detected?

Personnel taking swabs must be trained in doing so effectively. It is vital that all staff carrying out swabbing are doing so in a consistent uniform manner, as failure to adopt a consistent procedure will make trending of the data difficult. Sampling errors can be reduced by comparing results taken by different teams. A site map identifying facility layout, traffic flow and hygiene zoning areas, i.e., High Care/Risk, GMP physically segregated areas and within-area zones,

should help drive site selection. Both food contact and non-food contact surfaces must be sampled for *Listeria spp.*, the frequency of monitoring being dependent on the level of risk of cross-contamination, unless stipulated in legislation.

A cross-functional food safety team with knowledge of the plant's programs, processes, and practices should be established to develop a list of sampling sites. In addition to food production areas and equipment, the sampling scheme should also include processing aids (such as compressed air, ice, brine solution, water) and drain water (ANSES, 2017).

Data must be analyzed to identify harborage points and transfer vectors and trended to identify potential loss of hygiene control and trigger investigative and corrective action.

The timing of swabs makes a great difference to the interpretation of results if they are taken during production, or before or after cleaning. This information must always be recorded.

Swabbing should be carried out at different times depending on purpose of sampling, e.g.,

- Monitoring of controls or investigation: during production and post-hygiene
- To determine efficacy of hygiene protocols: post-hygiene
- To determine the most appropriate frequency of cleaning and disinfection: during production
- To identify or find *L. monocytogenes*: prior to cleaning and disinfection

Swabbing should be carried out using swabs appropriate to the geometry of the surface being swabbed. Sterile sponges are suitable for sampling large areas (e.g., 12 x 12 inches) and smaller "drumstick" swabs may be used for small or difficult-to access areas. Sponges and swabs should be moistened with an appropriate buffer solution containing an appropriate[1] neutralizing agent if the surface has been sanitized or disinfected (Table 1). A separate sponge or swab should be used for each distinct site.

For sponges, sampling should cover as large an area as reasonably possible using firm rubbing/abrasion to enhance the chances of finding organisms where biofilms may have established. For long pipelines or inaccessible assemblies, rinsing with a buffer solution and then testing the rinse sample is an acceptable practice. European Union (EU) Reference Laboratory guidance refers to a 1000-3000 cm^2 of open, flat areas being sampled.

Table 4. Neutralizing diluent which can be used in most situations - adapted from ISO 18593 (ISO, 2018)

Component	**Concentration (g/l)**
Sorbitan monooleate (Polysorbate 80)	30
Lecithin	3
Sodium thiosulfate	5
L-Histidine	1
Saponin	30
Peptone	1
Sodium chloride	8.5

US dairy industry guidance (DMI, 2015) recommends at least 100 x 100 cm for conveyors/tables. This will not be possible for harder to access points such as pipework. Templates for defining swabbing areas should not be used, as they can transfer *Listeria spp.* from one surface to another. Detailed information on the use of swabs and data to demonstrate the survival of low numbers of the target organism over the specified timescales between sampling and testing, should be available from the swab provider or laboratory.

Monitoring points must be reviewed regularly including in response to production changes or any incidents to ensure that new risks are identified and monitored. Sampling points should be varied over, e.g., a weekly or monthly period.

*Listeria s*wabbing is categorized into:

- Routine monitoring
- Investigation of environmental failure or a positive result in a finished product.

Routine Monitoring

The sampling plan should incorporate static, rotating, and random sites with planned sample numbers that take into consideration risks such as raw/RTE crossover, facility/equipment age and condition, history, and product type. Equipment and food contact surfaces must be sampled following cleaning and disinfection at critical points in the food production process to demonstrate that the hygiene program is effective.

To increase the probability of detecting a persistent strain, sampling should be performed during processing. EU reference laboratory guidance stipulates this should be "after at least two hours of production or at the end of production runs, i.e., before cleaning and disinfection". However, particularly in the UK, production runs may be as short as 30 min, with a clean down on changeover.

EU reference laboratory guidance states that "When foodstuffs entering the processing premises are raw or have been treated to decrease their microbial load (by pasteurization, microfiltration etc.), the food business operators (FBOs) should, as part of their HACCP plan, establish acceptable number of positive samples which can be set differently for the detection of *L. monocytogenes* on food contact surfaces and non-food contact surfaces."
Routine sampling should focus on:

- Production environment areas historically associated with *Listeria* spp. growth (e.g., hollow rollers on conveyors, gasket material around doors, hollow support structures, grease inside bearings, slicers, dicers, drip pans, condensate).
- The most critical areas of the plant including the area between any kill step and final packaging.

In addition, interfaces, transition areas, and barriers between raw and RTE areas should be sampled to verify the effectiveness of separation.
Non-food contact equipment should be carried out to determine whether *Listeria* spp. are present that may pose an eventual risk to the food, or for investigational purposes. Sampling non-production and transition areas may also help to assess the effectiveness of preventive controls. Routine sampling timing should be rotated to ensure monitoring across all days, shifts, plant areas, and zones. Varying timing to represent the entire production schedule and to capture periodic events will help in investigating any issues.

Investigative Sampling

Investigative swabbing should always follow a defined plan and have a pre-determined timescale. It should not be incorporated into routine monitoring, as this increases the costs and makes interpretation of the resultant data more difficult. Timing of swabbing is determined by the specific circumstance. For instance, investigative swabbing can take place from finished product to raw materials and follow production steps backwards to identify where the

contamination is occurring. Sampling should also be carried out when conditions are atypical, such as during audits, tours, construction, etc. Sampling should always be carried out if a drain backup or roof leak occurs. In addition, a procedure should exist for swabbing all new incoming equipment, and pre- and post-swabbing for construction. Sample collection personnel should have the freedom to sample additional sites based on observations.

Using Environmental Sampling Data

Listeria spp.- positive swab data must be used to effectively address deficiencies in hygiene control. Steps to doing so include:

- Reviewing historical site data trends, identifying any common factors
- Reviewing effectiveness of cleaning (e.g., presence of biofilms), schedules, methodologies, regime, level of dismantling, chemicals used and finished hygiene standards. The chemical provider can advise on best chemical to treat *Listeria* and to clean the affected areas.
- Introducing additional swabbing in areas not normally swabbed if required as part of the investigation. Set a timescale for additional swabbing/testing and set a review date to identify areas of concern or unrelated to the issue.
- Checking the integrity of High Care/Risk barriers to ensure that they are effective. Have there been changes to process or products e.g. new equipment, chemicals, building work, raw material or supplier changes. Modifications to processing equipment, review equipment condition, seal integrity, damage to pipework, and look for opportunity for harbourage by stripping the equipment right down.
- Carrying out traceability of product (including raw materials), review cooking temperatures, transfers, factory fabric, and review waste handling,
- Following up with further swabs to refine the search area and look into particular spots.
- Developing a production facility map showing hot spots where *Listeria* spp. are likely to found. This information should be used to target enhanced cleaning and disinfection, if necessary, divert production away from those areas, heighten staff awareness of the need to comply with stipulated working practices, cleaning methods, chemicals, or manage factory infrastructure changes. Attention should also be paid to food contact surfaces

adjacent to areas which may present a risk of cross-contamination to the product either directly or via aerosols.

Samples and Laboratories

All samples must be labelled with all the information necessary to clearly identify the sample. This may include:

- Date and time taken
- Description or ID of sample point
- Type of sample, e.g., pre-clean *Listeria*, etc.

Samples to be sent for analysis must be handled so that there is no impact on the levels of *L. monocytogenes* reported in the test results. A maximum time and temperature between sampling and testing should be set as part of the management procedure, or memorandum of understanding if testing is to be carried out by an external laboratory. It should be a shorter period where the product being sampled is not pre-chilled. Procedures must be implemented regarding the storage of samples prior to delivery to the laboratory, transportation to the laboratory and handling and storage within the laboratory. Times and temperatures of samples at each stage should be taken and records maintained. For example, UK industry guidance (CFA and BRC, 2006)requires that chilled food samples be kept refrigerated at 0-5°C, and not frozen, and be tested within 24 h of being taken.

EU reference laboratory guidance (EURL, 2011) states: "Transport the samples in a cool box (8.3) between 1 and 8°C. If necessary, store the samples at the laboratory at 3°C±2°C. Examine the samples as soon as possible, preferably not later than 24 h after receipt at the laboratory and in any case not later than 36 h after sampling, according to EN ISO 7218 Standard (clause 8.3, 3rd paragraph before end and the next paragraph on perishable products) (ISO, 2007). The length of time before analysis should be recorded and written in the analysis report."

Testing laboratories must demonstrate that they are only using legally-recognised accredited methods validated for use with the food or other material in question. They must also be engaged in continuous competency assessment (ring testing). Instructions for testing laboratories should be agreed in advance, such as whether initial tests are based on presence/absence or whether enumerations should be completed in parallel. If further analysis is specified, the laboratory must be advised that one must retain samples until a decision regarding further analysis.

Results should be reported as presence or absence of *L. monocytogenes* at the sampling location, indicating, if known, the size of the sampled area. Results are required by EU law to be trended, adverse trends acted on to address potential loss of control, and efficacy of corrective actions verified.

Costs

Product and environmental sampling are required by EU law, albeit at frequencies either determined by risk assessment or stipulated in legislation. The volume of food and environmental sampling by businesses increases where legislation does not penalize discovery of *Listeria* spp. or *L. monocytogenes*, irrespective of levels found. In the UK, major chilled food producers and their own label customers view extensive sampling as a necessary cost of doing business, delivering major benefits of ultimately protecting the consumer, reducing business risk from recalls, and protecting reputation, so driving business development and consumer confidence.

In the U.K., the leading dozen or so major chilled food producers spend upwards of £2.5 million per year on food and environmental sampling, being split approximately £1.5 million on food and £1 million on environmental sampling (personal communication, Chilled Foods Association estimates in 2019). The data resulting from these *ca*. 250,000 annual food and environmental samples are used to continuously track and trend control of both the production environment, production controls and shelf life, and is shared through a central industry database, enabling performance benchmarking of sites, companies and industry *Listeria* (both *Listeria* spp. and *L. monocytogenes*) control performance.

Notable points:

- Where regulatory structures are designed to encourage food business operators to actively seek out *L. monocytogenes* by carrying out programmed, regular, substantial sampling without penalizing discovery, the data stream can be used to verify and trend efficacy of hygiene measures, rapidly identify either harborage/colonization points or any trends towards loss of hygiene control.
- This activity results in demonstrable public health benefits, particularly where it is enforced commercially, as in the U.K. chilled prepared food sector.

8. Estimation of listeriosis cases resulting from the consumption of select frozen vegetables among elderly men (U.S.)

Table 5. Estimated number of listeriosis cases among elderly men resulting from consumption of uncooked frozen vegetables.

	A	B	C	D	E	F	G	H	I
	Product processed in US in lbs. per capita	**RACC^ in g/per serving**	**U.S. population in million**	**Total amount of product processed in lbs. * (10^6)**	**Total amount of product processed in grams * (10^6)**	**Servings consumed in the U.S. in millions**	**Servings consumed in the U.S. in billion**	**Estimated no. of illnesses (worst case)**	**Estimated no. of illnesses (best case)**
Frozen Vegetables	**USDA**	**FDA**	**US Census**	**(A*C)**	**(D*454)**	**(E/B)**	**(F) in billion**	**(G*3800/1000)**	**(G*400/1000)**
Spinach	0.72	85	328.2	236.3	107280.2	1262	1.262	4.79	0.50
Green Peas	1.25	85	328.2	410.25	186253.5	2191.2	2.191	8.32	0.87
Corn	6.85	85	328.2	2248.17	1020669.18	12007.87	12.007	45.6	4.80
Snap beans	1.92	85	328.2	630.14	286083.56	3365.68	3.365	12.7	1.34
Carrots	1.76	85	328.2	577.63	262244.0	3085.22	3.085	11.7	1.23
Total								83.11	8.74

Table 5 – Legend

A: Lbs. per capita (USDA)
https://www.ers.usda.gov/data-products/food-availability-per-capita-data-system/ /Accessed 30 May 2020

B: ^Recommended Amounts Customarily Consumed (RACC) (g/serving) (FDA)
https://www.fda.gov/media/102587/download /Accessed 30 May 2020

C: Total U.S. population in millions (2019) U.S. Census, Population:
https://www.census.gov/newsroom/press-releases/2019/popest-nation.html /Accessed 30 May 2020

D: Total lbs. of frozen product processed in the U.S.

E: Total gm. of frozen vegetable processed in the U.S.

F: Total no. of frozen vegetable servings consumed annually in the U.S. (million)

G: Total no. of frozen vegetable servings consumed annually in the U.S. (million)

H: Total no. of illnesses among elderly men resulting from consumption of frozen vegetable in the U.S. (worst case scenario when uncooked - assume 3800 cases/10^{12} servings). Assumptions are based on EFSA Scientific Opinion (Koutsoumanis et al., 2020)
https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2020.6092 /Accessed 10 Jun 2020

I: Total no. of illnesses among elderly men resulting from consumption of frozen vegetable in the U.S. (best case scenario when uncooked - assume 400 cases/10^{12} servings). Assumptions are based on EFSA Scientific Opinion (Koutsoumanis et al., 2020)
https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2020.6092 /Accessed 10 Jun 2020

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